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THE APPARENT METAMORPHOSIS AND RELATED DEFENSE REACTIONS OF HAEMOCYTES IN THE LAWN ARMYWORM, *SPODOPTERA MAURITIA ACRONYCTOIDES* (Guenée)

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ABSTRACT

Physiopathological studies involving the lawn armyworm, Spodoptera mauritia acronyctoides *(Guenée) and two pathogens found associated with it in Hawaii, a nuclear polyhedrosis virus (NPV) and a microsporidian,* Vairimorpha, *have revealed that the haemocytes in this host insect change in quantity, form, and activity with growth and development during the larval and early pupal stages. The blood cell changes in whole haemolymph samples collected from larvae and pupae were observed to follow a timely pattern with aging as growth and development progressed, indicating an apparent metamorphosis of haemocytes in this moth. Larvae which are about 15 days old are characterized by individual prohaemocytes, cystocytes, adipohaemocytes, and spherule cells that multiply and increase in quantities with growth and aging. More forms of haemocytes may be observed with the increases in quantities, until plasmatocytes, oenocytoids, vermiform cells, granular haemocytes, and podocytes may also be differentiated. The multiplication and differentiation are followed by aggregations and fusions of plasmatocytes and granular haemocytes, and agglomerations of these and other haemocytes form complexes and nodules, respectively. Prior to pupation the individual and complexes of haemocytes begin vacuolating and granulating, these processes believed to be indicating the degeneration of tissues. During these cellular processes, large numbers of small vacuolelike globules with some larger blebs are produced along with increasing quantities of dark granules. Phagocytosis of tissue fragments is observed in the individual and complexes of haemocytes, while both phagocytosis and encapsulation are believed to take place during nodular formation. Hyaline haemocytes, other forms of pleomorphic plasmatocytes, and intermediate forms of haemocyte transformations are also observed during this late larval period. In the early pupal stage, the degeneration continues until the individual and complexes of haemocytes become filled and swollen with globules, granules, and phagocytozed tissue fragments, and some cells begin disrupting. By the time pupae are three days old, the phagocytozed tissues, along with all the other materials that have accumulated within the cells, transform into highly refractive spheres or balls. These spheres dominate the haemolymph while only outlines and remnants of blood cells remain. Many phases in the apparent metamorphosis of haemocytes were accentuated during comparative studies between armyworms exposed to treatments of pathogens and other stress situations and untreated, control armyworms. Armyworms appearing normal that are under stress and infected armyworms demonstrate clearly the progressive changes of haemocytes that take place during the apparent metamorphosis of these cells. However, in these specimens the changes are premature, occurring earlier in the growth and developmental periods of the armyworm than in control specimens. The precocious developments are considered to be part of the defense reactions of* S. mauritia. *This and the apparent metamorphosis of haemocytes are discussed from different viewpoints.*

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INTRODUCTION

Since the late nineteenth century, an abundance of research involving insect blood cells has been reported. In addition to studies concerning the morphology and classification of haemocytes, many investigations have been concerned with defense reactions and immunity of host insects to parasites and pathogens. Salt (1963), Poinar (1969), and Shapiro (1969) have compiled extensive reviews on the immunity of insects to parasitic worms, while Huff (1940), Steinhaus (1949), Wagner (1961), Stephens (1963), Briggs (1964), and Heimpel and Harshbarger (1965) are some of the authors who have emphasized their reviews on insect immunity and microbial infections. The activities of haemocytes in immune responses are associated with phagocytosis, cell transformations and melanization. Basically, these are regarded as cellular defense reactions, but Salt (1963) and Shapiro (1969) consider melanization to be a humoral reaction.

Wittig (1962) classifies activities of haemocytes in pathology as histological, cytological, or cytochemical. According to this classification, changes in total haemocyte counts (THC) as reported by Wheeler (1963), Laigo and Paschke (1966), Wittig (1965, 1966), Shapiro (1967), and Bahadur and Pathak (1971), as well as changes in differential haemocyte counts (DHC) as reported by Wittig (1966) and Nappi (1970*a*, *b*), are histological changes. Some examples of cytological activities are phagocytosis (Wittig, 1965, 1966, 1968; Werner and Jones, 1969), cell aggregation, and encapsulation during parasitization (Van Den Bosch, 1964; Salt, 1965, 1966; Poinar et al., 1968; Nappi and Stoffolano, 1971, 1972), and wound closures by coagulations (Beard, 1950; Gregoire, 1951, 1974). Melanizations as reported by Rizki (1957*a*, *b*, 1960) and Nappi (1970*b*) are cytochemical changes.

Interestingly, Arnold (1974) has organized the many varied processes of haemocytes, as reported over the years, into haemocyte functions and haemocyte activities. In the first category he has acknowledged four basic functions: (1) phagocytosis of small particles the size of microorganisms, (2) encapsulation of large, foreign objects such as parasites and parts of parasites, (3) coagulation of the blood by cellular agglutination and/or by contributing to plasma precipitation, and (4) storage and distribution of nutritive materials. Included in the haemocyte activities category are such processes as healing of wounds, nodule formation about clumps of foreign particles, the removal of lysed tissues, membrane (tissue) formation, control of hormone action and the regulation of growth, detoxication of chemicals, melanization of cuticle, and other processes which may involve one or more of the four acknowledged functions.

While studies concerning haemocytes have been extensive, there still remains much confusion in the information accumulated for these insect blood cells, particularly in the areas of haemocyte reactions and processes. The role of these cells in insect immunity is unclear, as are the reasons for some of the activities themselves and the factors that are involved in their control. Salt (1970), points out that even for the well-described and accepted cellular defense reaction, phagocytosis, information is lacking, especially regarding its selectiveness and the mechanisms or stimuli involved in its initiation. Wittig (1962) suggests that many of the anomalies that occur in haemocyte studies involving microbial infections are apparently enhanced by the difficulty in separating defense reactions from disease reactions. She classifies phagocytosis as a defense reaction and considers both defense and disease reactions of haemocytes as abnormal processes. There are many authors in addition to those previously cited who indicate that the present state of knowledge about haemocytes is inadequate and that there is a great need for more research involving insect blood cells. However, it is apparent from the many inconsistencies in studies concerned with haemocytes that these cells are dynamic and capable of changing and reacting to environmental factors and other influences. Therefore, any study concerning these cells should be undertaken with the strictest possible control. The parameters and conditions should be specified in detail in reports of results of any aspects of studies of haemocytes to ensure reproduction and consistency by other investigators.

This is a report of studies and observations of haemocytes in blood samples from larval and early pupal stages of the lawn armyworm, *Spodoptera mauritia acronyctoides* (Guenée), during physiopathological investigations of the host insect and two pathogens found associated with it in Hawaii—a nuclear polyhedrosis virus (NPV) and a microsporidian, *Vairimorpha.* Techniques were used to maintain conditions whereby physiological reactions and effects of the host could be attributed to varying levels of exposures to pathogens or to other applied or controlled factors. During the studies, processes that ordinarily would not be noticed were observed in the haemocytes of the lawn armyworm through comparative studies between control and treated specimens. Changes and processes observed resembled an apparent metamorphosis of lawn armyworm haemocytes. The different types or categories of haemocytes are described in both control and treated specimens according, basically, to the classification proposed by Jones (1962).

MATERIALS AND METHODS

Synxenic per os inoculations of fourth instar (eight-day-old) larvae of *S. mauritia* with varying levels of pathogens were carried out according to the methods described by Takei and Tamashiro (1974). The methods essentially consisted of maintaining aseptic techniques while applying axenically prepared suspensions of pathogens on the diet media of axenically reared host insects. Most of the rearing and observational studies were carried out with 8-dram vials that contained an oligidic diet medium devised specifically for the host. Within the vials, synxenic per os inoculations were achieved by applying the suspensions of pathogens directly on the diet medium with a hypodermic needle and syringe. Comparative studies were made of inoculated and control lawn armyworms to determine the effects of the treatments.

The haemocytes were examined *in vitro,* in undiluted, unfixed, wet mounts of whole hemolymph by phase contrast microscopy. Air dried blood smears fixed with alcohol and stained with Giemsa also were examined. The blood cells of both treated and control larvae and early pupae were studied and compared immediately after collection.

Hemolymph samples were collected as described by Takei and Tamashiro (1975). From larvae, the collections were made by punctur-

ing the regions just above the bases of their prolegs with a fine needle. Pupae were bled by carefully puncturing the anterior ends either dorsally or ventrally. A small crystal of phenylthiouria (PTU) was placed in each collecting vessel as a precautionary measure against melanization of the sample. (However, melanization did not appear to be an interfering factor in axenic haemolymph samples.)

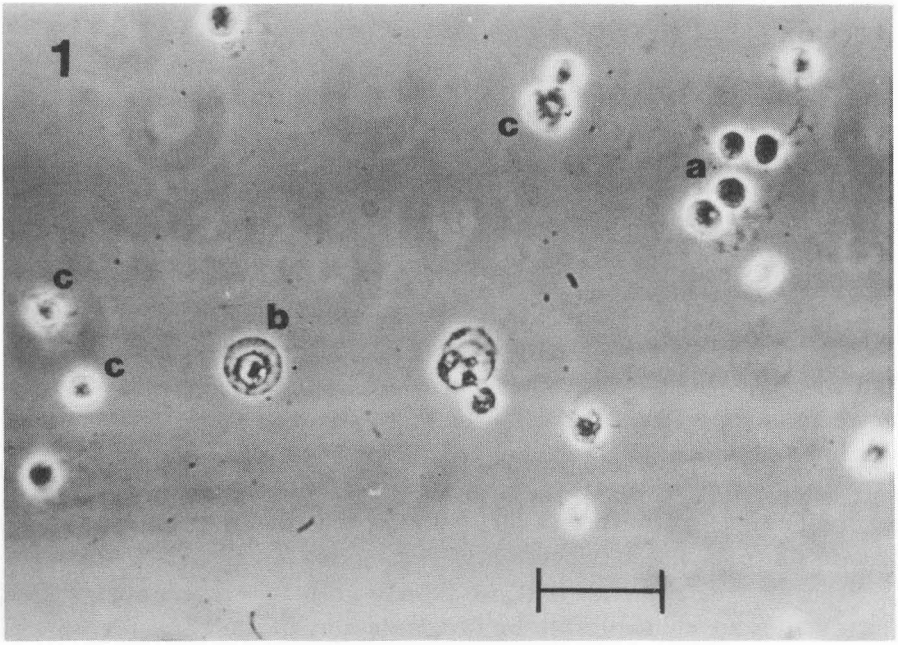
Included in the studies were lawn armyworms inoculated with sublethal concentrations of pathogens, armyworms surviving normally lethal concentrations, and armyworms that were infected to varying degrees and intact enough to be bled. The pathogens, *Vairimorpha* and NPV were applied singly and in combinations. Comparative studies also were made between larvae reared axenically on diets with and without formaldehyde, to determine the effects of the antimicrobial additive on the host insect.

RESULTS

Haemocytes in Control *S. mauritia*

The studies of haemocytes in control *S. mauritia* specimens revealed that these blood cells change with growth and development. The haemocytes were observed to vary morphologically, quantitatively, and according to a definite pattern of processes with time and aging of the host insect. Figures 1 and 2 are phase contrast photomicrographs showing haemocytes in armyworms that ranged in age from 13 to 17 days after eclosion. During this period of armyworm development, the haemocytes appear in relatively low numbers and generally are spherical and individual prohaemocytes, cystocytes, adipohaemocytes, and spherule cells. Prohaemocytes and adipohaemocytes are the most frequently observed, and as the larvae approach 20 days, these same types of haemocytes become more abundant, and plasmatocytes, many of which are apparently from prohaemocyte transformations, begin to appear

Figures 1, 2. Free-flowing, independent haemocytes in blood samples from armyworms that were 13 to 17 days old. Phase photomicrographs, 400x. Figure 1, haemocytes, generally spherical, entire, individual, and not too abundant; more noticeable ones are: **a**, group of prohaemocytes; **b**, cytocytes; **c**, adipohaemocytes. Figure 2: **d**, spherule cell. Bar = 10 μm in Figure 1 (same scale may be applied to all Figures).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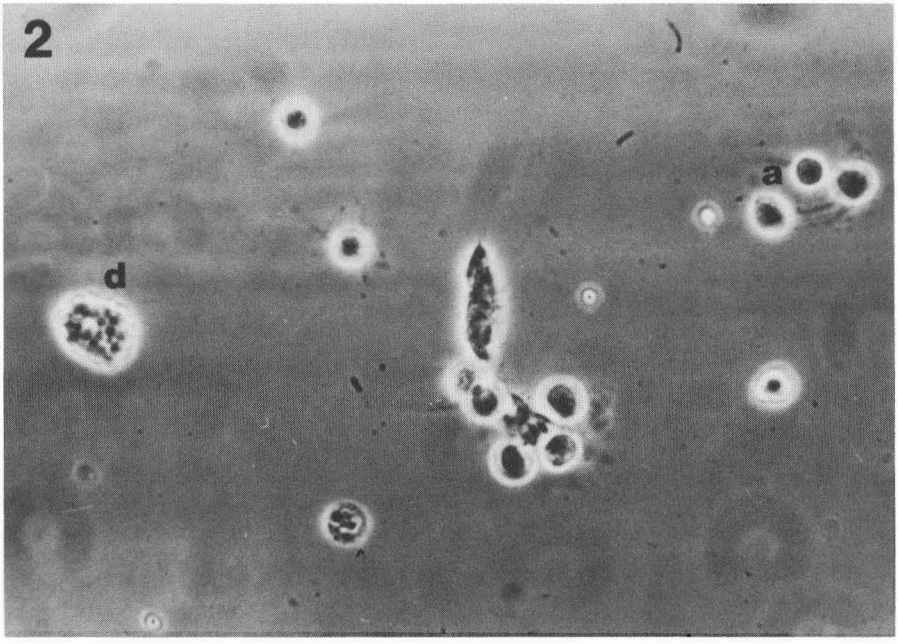
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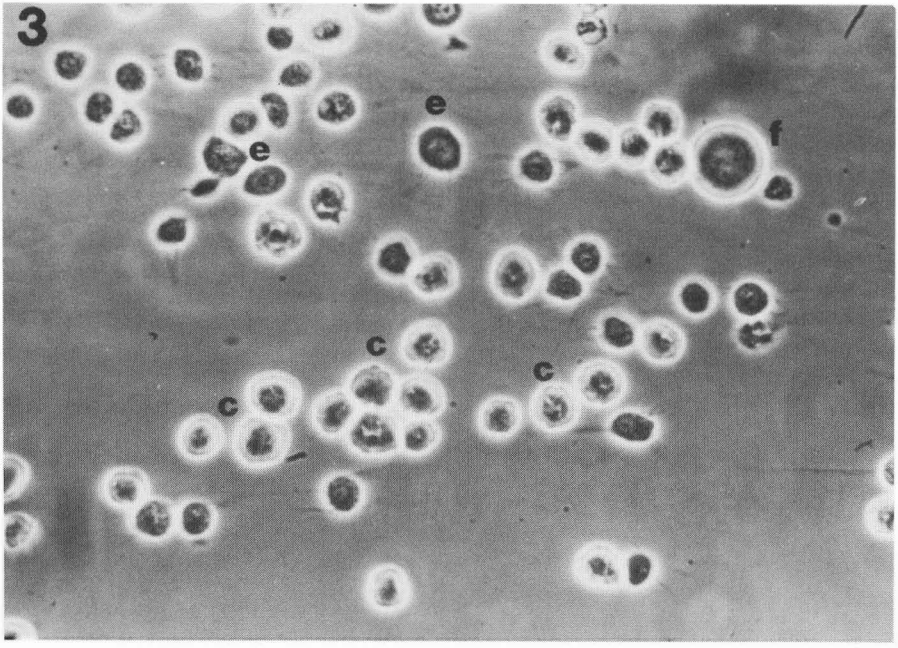
and increase quantitatively (Figure 3). An oenocytoid is also apparent, adding to the greater variation in blood cell types.

In Figures 4 through 7, haemocytes in 21- to 26-day-old larvae are shown. Some of the plasmatocytes, which are usually described as being pleomorphic, appear to be altering to fusiform shapes, while an abundance of other plasmatocytes may be seen with extruded pseudopodia and threadlike cytoplasmic extensions (filopodia) and appear to be clumping or agglomerating to form nodules. Still other plasmatocytes appear to be aggregating in large numbers and fusing together. Lamellocytes, which are extremely flattened and have large hyaline cytoplasms as described by Rizki (1957*a*, *b*, 1962), also are evident. Some of these cells are believed to be the hyaline coagulocytes described by Grégoire (1951, 1974). Generally, the haemocytes that are the most abundant during larval ages beyond 20 days are plasmatocytes of varying forms, most of which appear to be aggregating and/or agglomerating. Vermiform cells, granular haemocytes, and podocytes also begin to appear in haemolymph during this larval period.

From the late larval (26- to 28-day-old) to early pupal stages, haemocytes are characterized by processes that appear to be the start of cellular degeneration. Figures 8 through 13 show haemocytes for these stages of development. During the late larval period the cell outlines

Figure 3. Haemocytes in armyworms about 20 days old. Phase photomicrograph, 400x. Haemocytes apparently increasing in numbers with age; **e**, plasmatocytes (apparently from transforming prohaemocytes); **f**, oenocytoid.

Figures 4–7. Haemocytes continuing to change and increase in numbers in armyworms 21 to 26 days old. Haemocytes, mainly plasmatocytes, are aggregating, fusing together, and agglomerating. Phase photomicrographs, 400x. Figure 4: **g**, elongated threadlike cells, vermiform cells; **h**, haemocytes clumping to form independent nodular groups. Figure 5: **i**, plasmatocytes; **j**, haemocyte with flattened, large hyaline cytoplasm (lamellocyte), Rizki, 1957*a*, *b*, 1962; hyaline haemocyte (coagulocyte), Grégoire, 1951, 1974; **k**, elongated fusiform plasmatocyte; possibly intermediate of vermiform cell or side view of lamellocyte; **l**, believed to be intermediate form between cystocyte and hyaline haemocyte (lamellocyte); **m**, lysing or disintegrating granular haemocyte (possibly oenocytoid). Figure 6: **n**, more haemocytes clumping into apparent nodules (note filopodial extensions); **o**, plasmatocytes fusiform in shape (note streaks that are believed to be plasma reactions associated with extensive cytoplasmic filopodia throughout Figures 5 and 6). Figure 7, abundance of plasmatocytes aggregating and fusing: **p**, aggregate of 4 prohaemocytes included in photomicrograph; **q**, hyaline haemocytes apparently associated with the aggregations.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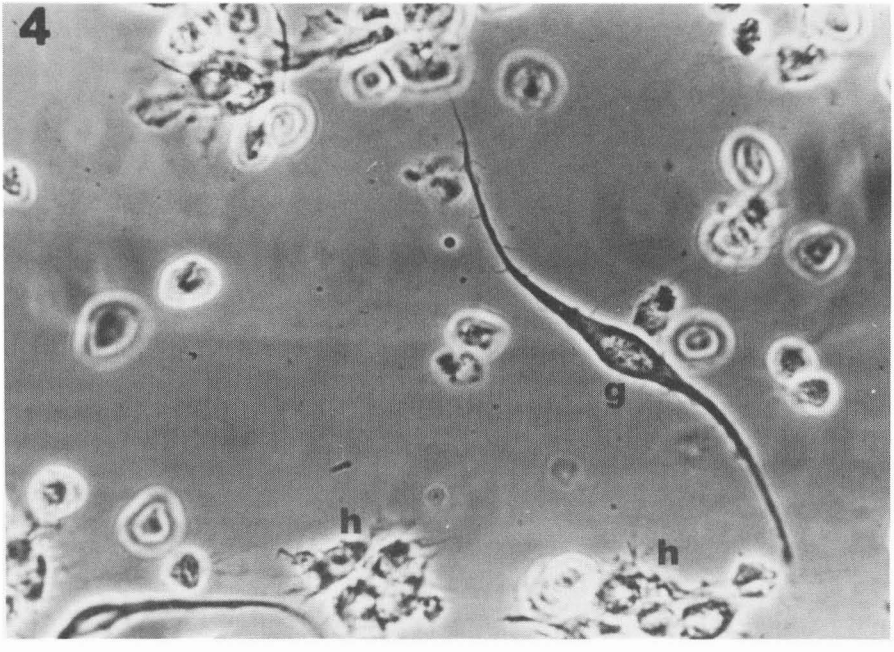
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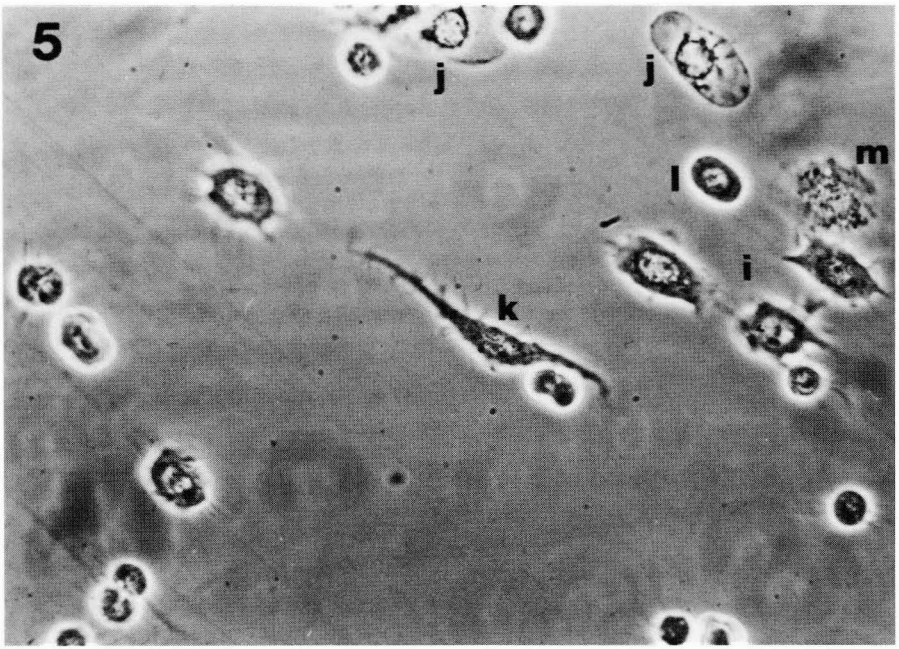
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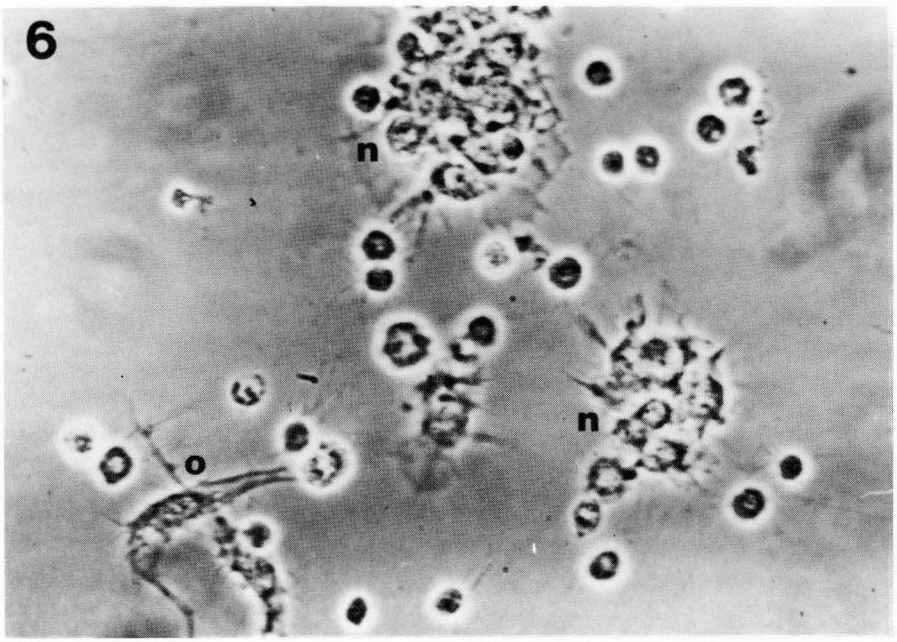
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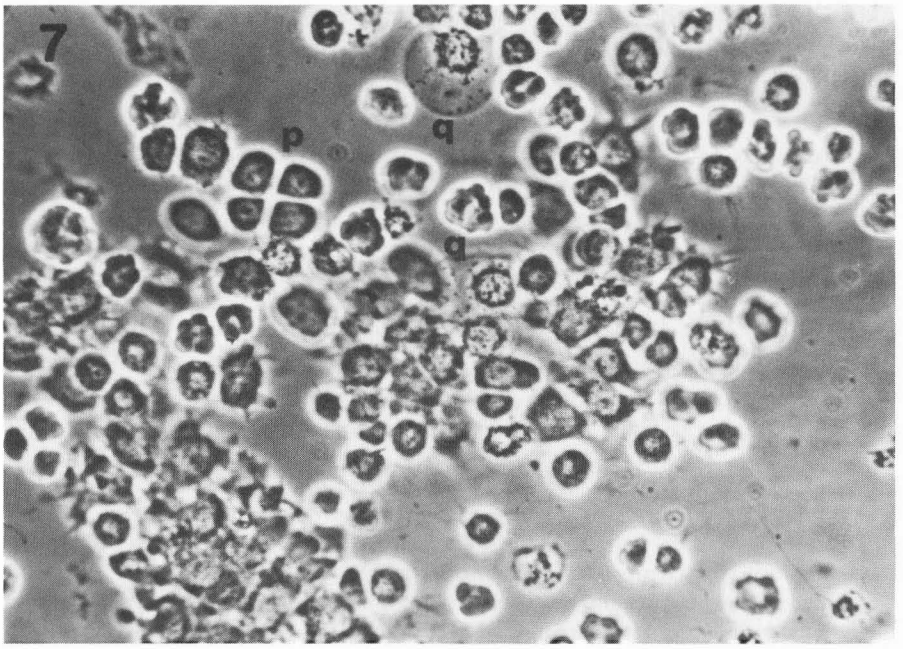
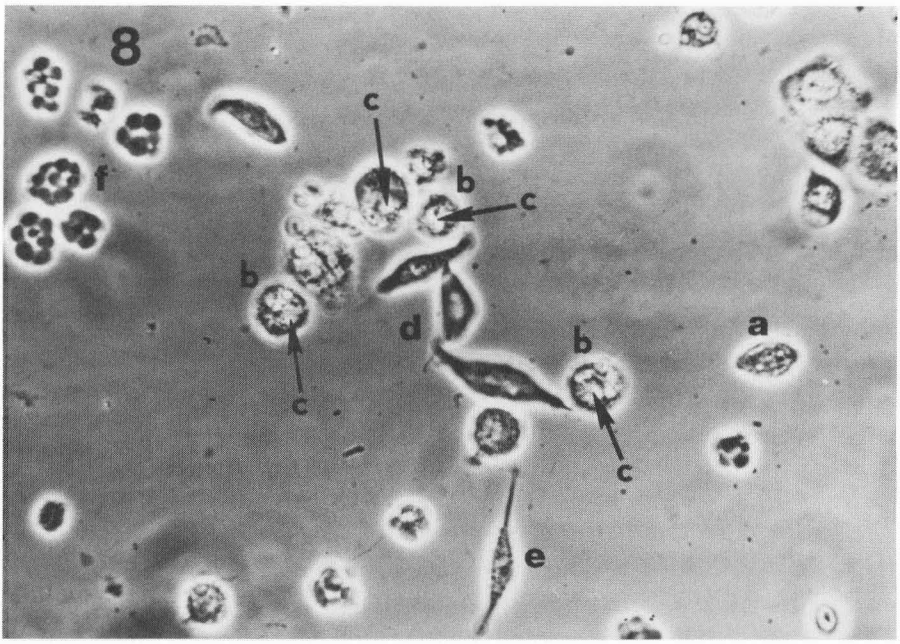


and structures still are basically maintained, but many individual haemocytes may be observed forming vacuoles, dark granules, and vacuolelike globules in the cytoplasms and nuclei, and these new forms begin to obscure the cells. The haemocytes, mainly plasmatocytes, that have aggregated or fused to form large complexes during earlier larval growth periods also continue altering themselves. These complexes begin to show dark granulations and vacuolations consisting of small spherical globules. Small, round, clear, vacuolelike, highly refractive droplets appear first within the cytoplasms of individual and large complexes of blood cells and then appear to increase more in numbers than size until they also obliterate the nuclei of the cells. The processes are believed to be associated with the early changes and activities in haemocytes that anticipate metamorphosis and pupation in *S. mauritia.* All the types of haemocytes that can be differentiated during this period of development appear to be vacuolating or forming vacuolelike globules to varying degrees, depending on cell type, but the process is evident first in plasmatocytes. Furthermore, most of the haemocytes are believed to be phagocytes forming small, vacuolelike globules, larger spherical blebs, and dark granules when metamorphic hystolysis of

larval tissues is believed to begin. These activities occur in much the same way as described by Grégoire (1974) for haemocytes exposed to anticoagulants and X rays. Haemocytes phagocytozing larval tissues, then, also characterize this period while other cells may be observed changing to different forms. Fusiform plasmatocytes appear to be altering to spindle haemocytes while other plasmatocytes may be observed with long, filamentous extensions on opposite ends of the cells.

The vacuolations and granulations in many of the haemocytes also are believed to be the start of the transformation of blood cells to granular haemocytes as described by Whitten (1964) for *Sarcophaga bullata* (Parker). Moreover, she has reported that these granular haemocytes take part primarily in phagocytosis while accompanying histolysis and formation of new tissues during the prepupal and pupal

Figures 8–13. Haemocytes of the late larval period (26- to 28-day-old larvae). Degeneration of haemocytes apparently starting. Phase photomicrographs, 400x. Figure 8, individual haemocytes undergoing changes and alterations: **a**, individual haemocyte showing the formation of small vacuolelike globules; **b**, other individual haemocytes also vacuolating with formation of globules, as well as some phagocytozing; **c**, round, highly refractive bodies within cells, believed to be phagocytozed tissue fragments; **d**, fusiform plasmatocytes; **e**, precursory forms of spindle-shaped plasmatocytes (also in Figures, 9, 11, and 12); **f**, adipohaemocytes not fusing or agglomerating, losing refractiveness and apparently on the verge of degenerating and fragmenting (also in Figure 10). Figure 9, haemocytes in same state of development as those shown in Figure 8; **g**, haemocytes with long, filamentous extensions on opposite ends of the cells. Figure 10, haemocytes showing processes of changes and alterations; **h**, abundance of vacuolelike globules and dark granulations surrounding tissue fragments in granular haemocytes; **i**, engulfed larval tissue fragments; **j**, individual large, vacuolating, granular haemocyte phagocytozing; **k**, smaller, individual granular haemocytes (**h**, **i**, **j**, **k**, also in Figure 13); **l**, apparently disintegrating haemocyte (granular) voiding contents. Figure 11: **m**, complexes of phagocytozing granular haemocytes (also in Figure 13); **n**, nucleus being pushed to periphery of complex (also in Figure 13); **o**, cytoplasmic pseudopodial extensions; **p**, granular haemocyte with apparently clear, fluid material surrounding cell (coagulocytelike). Note similar processes from different regions of the cell complexes. Figure 12: **q**, nodule, highly refractive and yellow, appearing to encapsulate tissue fragments; **r**, haemocyte with inclusions partly extruded into plasma; **s**, hyaline haemocyte nearly devoid of inclusions and swollen due most likely to imbibition; **t**, haemocyte expanding through vacuolations and formations of spherical blebs. Figure 13: **u**, cellular exudates, plastidlike; **v**, remnants of hyaline haemocytes with inclusions nearly completely extruded; **w**, phagocytozing haemocytes apparently fusing.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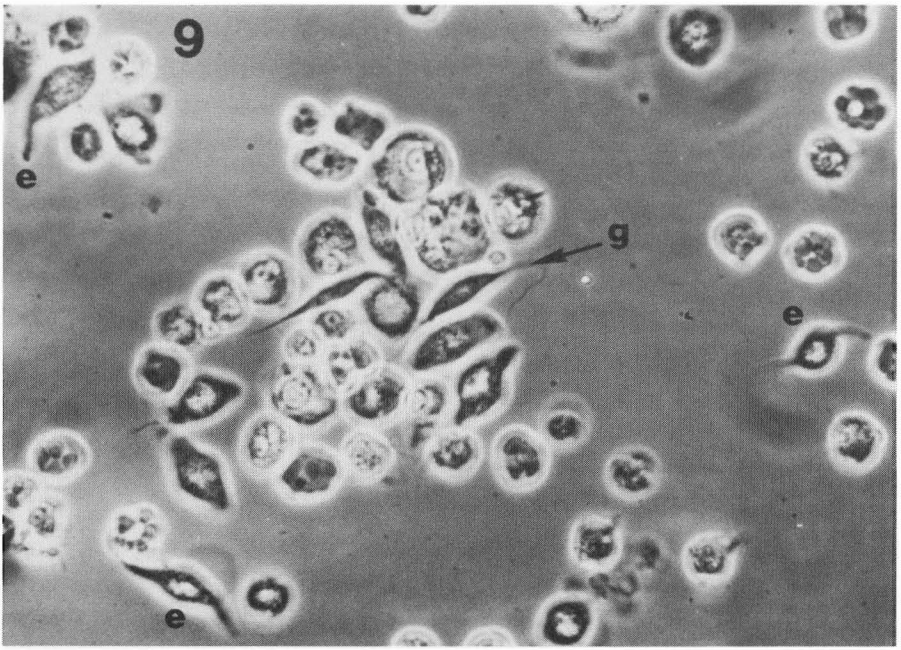
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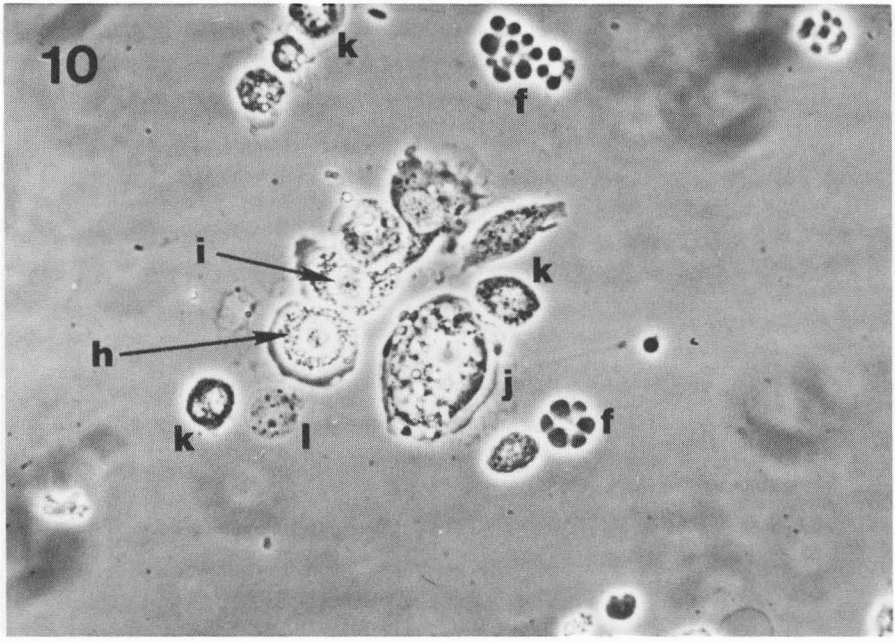
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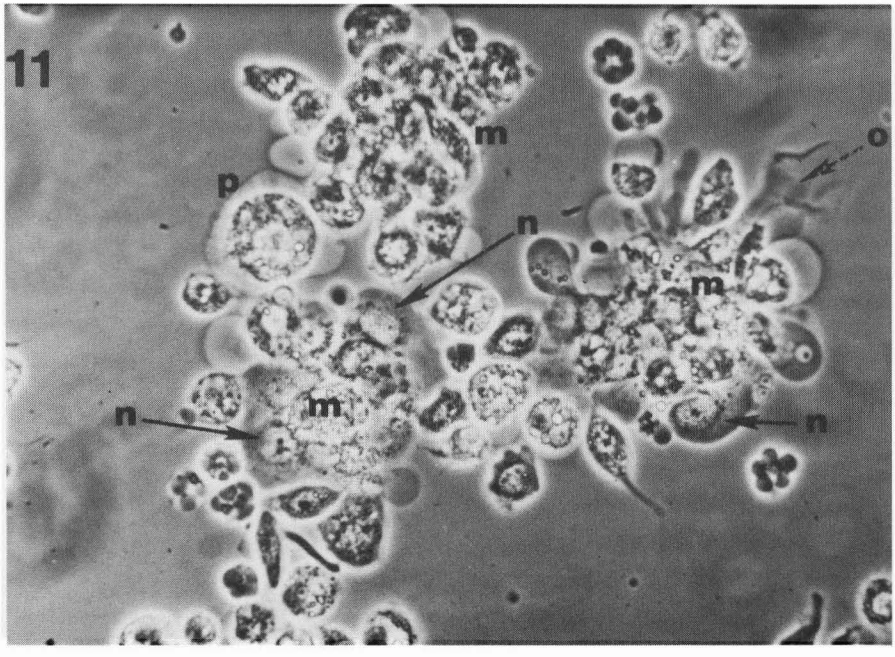
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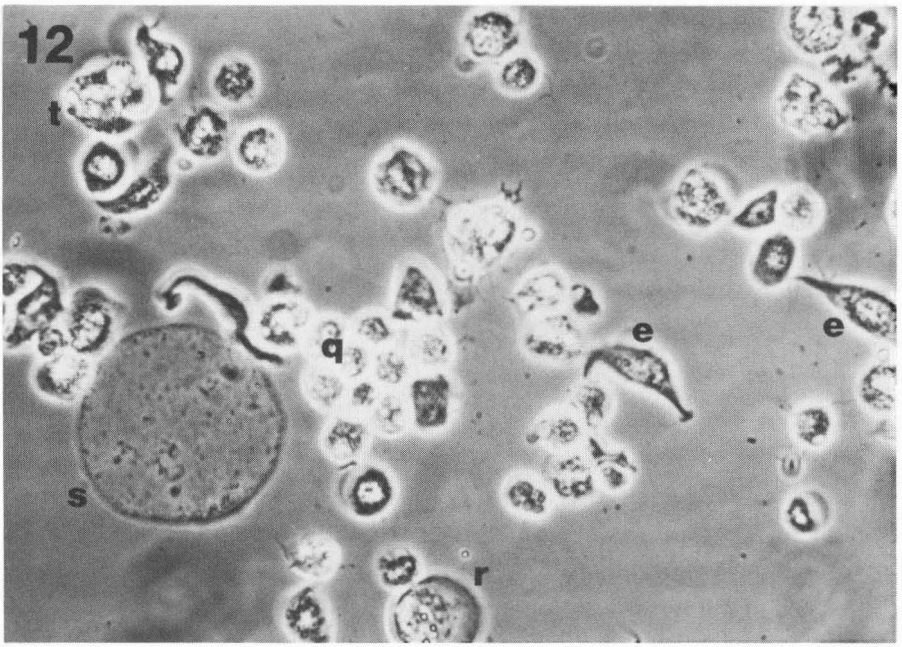
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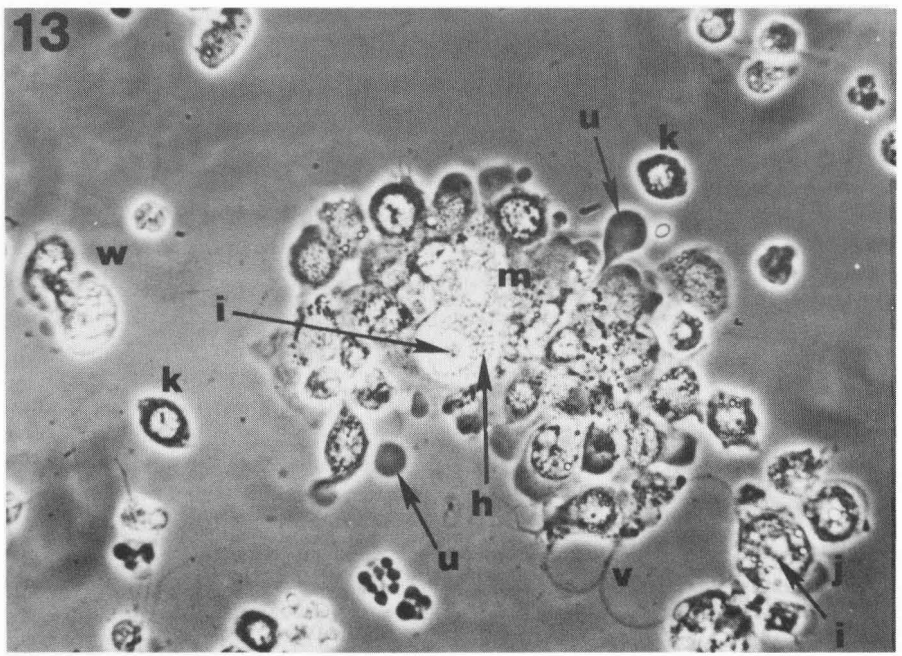
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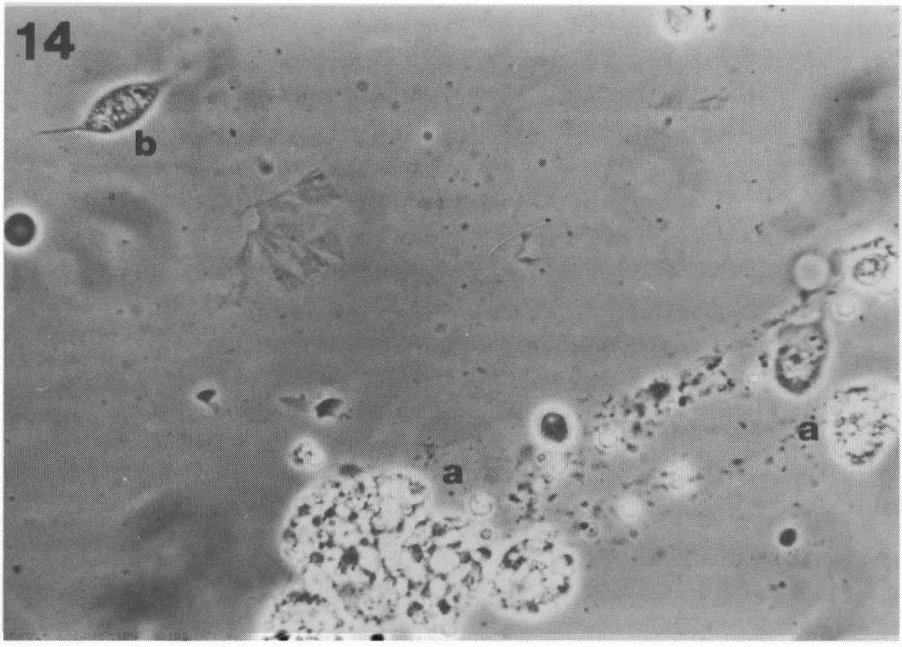
stages. Highly refractive, yellow bodies with occasionally reddish centers are believed to be fragments of larval tissues and may be seen phagocytozed within both individual and complexes of granular haemocytes.

As phagocytosis proceeds, the vacuolelike globules and dark granules, while increasing in numbers, continue to surround the larval tissue-fragments that are engulfed within individual haemocytes and haemocyte complexes. The phagocytozing haemocyte complexes are believed to parallel the multinucleate granular haemocytes in *S. bullata* as described by Whitten (1964) during similar phases of growth and development for this fly. While phagocytosis is taking place, fluid substances appear to be exuding from the cytoplasms of individual and complexes of granular haemocytes into the plasma, and these cytoplasmic extensions appear to be engulfing tissue fragments. Hyaline haemocytes appear at this time to have almost entirely expelled their cellular inclusions, and some remnants of these haemocytes may be seen during the prepupal stage. As the apparent processes of degeneration and metabolism of individual and complexes of haemocytes continue, many of the phagocytozing cells become highly refractive, and appear basically light yellow when viewed under phase contrast microscopy. Agglomerates of haemocytes that have apparently encapsulated larval tissues while forming nodules show this high refractivity particularly well. Adipohaemocytes are among the cells that do not take part in agglomerations and fusions but seem to be on the verge of degenerating and fragmenting. Other individual haemocytes continue to change, also seemingly toward disruption and disintegration.

The changes in haemocytes accompanying the development of *S. mauritia* in the pupal stage is shown in Figures 14, 15, and 16, photomicrographs of haemolymph samples from 1-day-old pupae. During the pupal stage, individual and complexes of granular haemo-

Figures 14, 15, 16. Haemocytes in 1-day-old pupae. Phase photomicrographs, 400x. In this stage, haemocytes continue phagocytosis; **a**, individual and complexes of granular haemocytes continuing phagocytosis and degeneration, some with disruption and expulsion of inclusions; cell outlines completely filled with globules, dark granules, and phagocytozed tissue fragments; some nuclei apparently remain intact with evidences of granulations; **b**, spindle-shaped plasmatocyte; **c**, granules and globules being extruded into the plasma by the degenerating cells; some light cytoplasmic pseudopods evident; **d**, cells apparently not taking part in phagocytosis and degeneration; **e**, granules and globules already extruded in plasma.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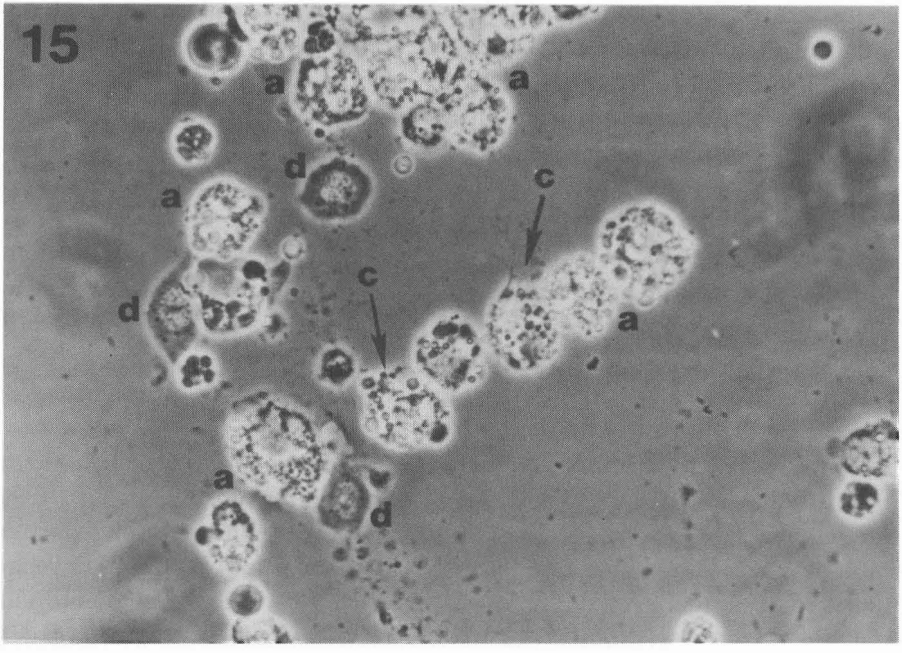
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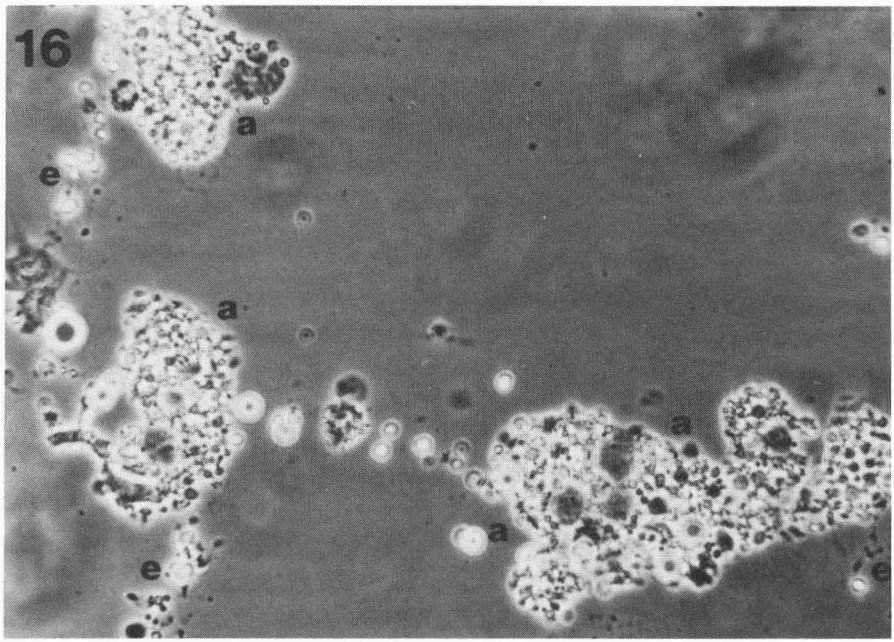


cytes continue phagocytosis. Tissue fragments can be seen being engulfed and surrounded by the phagocytes while vacuolelike globules continue increasing markedly and the degeneration of the cells becomes more obvious. Some cells have disrupted, leaving granules, globules, and spherical blebs among other materials in the plasma (Figure 14). The globules now appear to be very much like virus polyhedra in refractivity, but unlike virus polyhedra they do not dissolve in a 10 percent solution of potassium hydroxide (KOH) and are not proteinaceous. The globules are more lipidlike and are virtually unaffected by the base treatment. They are believed to be fat vacuoles. The dark granulations within the degenerating cells continue to increase in quantity, becoming more prominent. Occasionally they are observed being expelled into the plasma.

Spindle-shaped haemocytes with short filamentous extensions on opposite ends of the cells and with inclusions on opposite sides of the nuclei also are observed. These cells apparently do not take part in phagocytosis, and are characteristic of the pupal stage. They are believed to originate from transformations of special forms of individual plasmatocytes. Some precursory spindle plasmatocytes were des-

cribed earlier. They are believed to be first observed in the late larval period and are fusiform.

Figures 17, 18, and 19 are photomicrographs of haemolymph samples from 3-day-old pupae. Phagocytosis has progressed to such an extent that many tissue fragments have been engulfed by the haemocytes and completely fill these cells. The haemocytes, filled with tissue fragments, apparently form spheres or balls as described also by Whitten (1964) for *S. bullata.* Large, highly refractive spheres of tissue fragments dominate the photomicrographs, but outlines and remnants of haemocytes are still visible. Among the haemocyte structures capable of being distinguished is the nucleus. Some spheres of tissue appear to be starting the formation of new tissues while fragmenting. Histolysis and phagocytosis are believed to be continuing within these spheres of haemocytes and tissue fragments, and they may possibly provide the metabolic energy necessary for the activities involved in metamorphosis.

Further changes most likely continue during the remainder of the pupal stage with continuation of histolysis and differentiation of new tissues. Haemolymph studies beyond the 3-day-old pupal stage and through the adult stage, however, were not conducted in this work, because complementary studies involving haemolymph protein analyses, as reported by Takei and Tamashiro (1975), also were not carried beyond this point in the pupal stage.

Haemocytes in Armyworms Subjected to Treatments of Pathogens

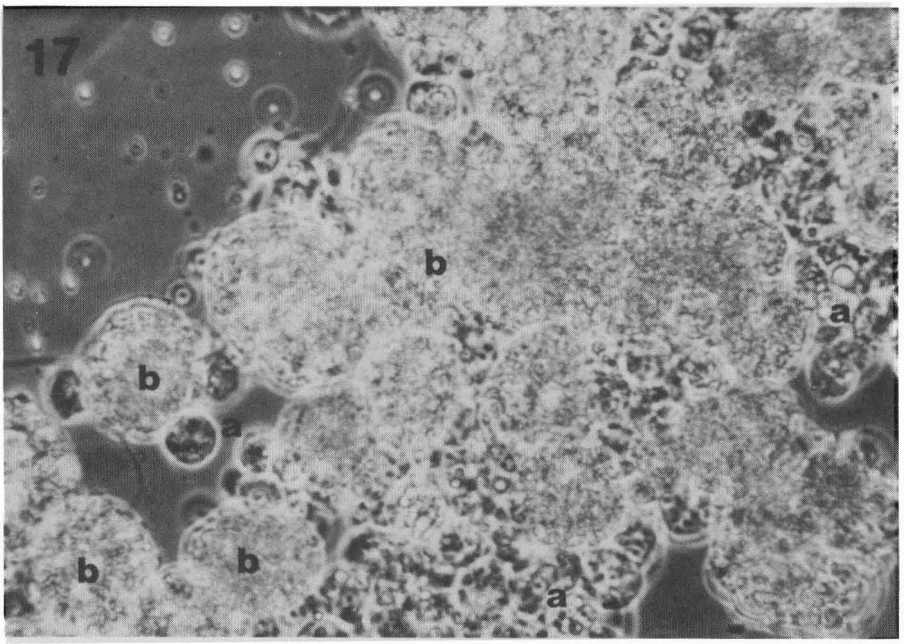
Among inoculated armyworms, those that withstood and survived exposures to pathogens provided haemolymph samples in which changes in haemocytes were obvious and easily identified. Moreover, these armyworms were intact since they were not overwhelmed with infections caused by applied pathogens and had no visible indications that their tissues and cells had been attacked, or damaged. Because of this they could be bled as well as the control larvae, giving haemolymph samples in volumes suitable for examination.

The haemocytes in these armyworms appeared, generally, to be more mature or at a more advanced state of development than haemocytes in control or untreated larvae of the same ages. Some haemocytes were observed aggregating and fusing together, agglomerating, granulating, forming vacuolelike globules, and phagocytozing tissue fragments earlier in the larval stage than what was usually observed in untreated,

control larvae. Figures 20, 21, and 22 are photomicrographs of haemocytes from 19-day-old armyworms that were treated with *Vairimorpha* and NPV simultaneously at levels not causing true infections by either of the pathogens alone, and Figures 23 and 24 are of haemocytes in control armyworms of the same age. The haemocytes in the treated larvae show marked differences from haemocytes in control larvae, and the haemocytes from treated larvae can be seen clumping to form nodules that apparently have encapsulated larval tissue fragments, seemingly as part of preparation for pupation. This haemocyte activity is not evident at all in the controls, and it is usually observed in untreated larvae when they are older than 21 days. Advanced forms of individual haemocytes, such as fusiform plasmatocytes, plasmatocytes with many fine cytoplasmic extensions, lamellocytes (hyaline haemocytes), vermiform cells, and podocytes, may be observed in the haemolymph of treated armyworms. But these advanced forms may not be observed in haemolymph of control armyworms, which usually have fewer haemocytes that are mainly prohaemocytes, adipohaemocytes, and cystocytes (see Figures 20–24). These advanced developments in haemocytes were obsered in armyworms that showed no ill effects or symptoms of infections caused by treatments of pathogens whether they were of doses of NPV alone, *Vairimorpha* alone, or combinations of the two pathogens. This indicated that the advanced developments were due to the effects of stress produced by the treatments.

Figures 17, 18, 19. Haemocytes in 3-day-old pupae. Phase photomicrographs, 400x. Phagocytosis and cell degeneration have progressed to an extent that highly refractive, golden bodies of tissue fragments and inclusions completely fill cell outlines, forming spheres or balls that replace the small vacuolelike globules and larger spherical blebs and dominate the photomicrographs. In some areas remnants of haemocytes and complexes of haemocytes are still discernable; **a**, **b**, highly refractive balls or spheres of phagocytozed tissue fragments and inclusions; **c**, early phase of sphere formation; **d**, fragmentation of spheres as histolysis continues with metamorphosis.

Figures 20, 21, 22. Haemocytes in 19-day-old armyworms, exposed simultaneously to sublethal doses of *Vairimorpha* and NPV. Phase photomicrographs, 400x. Note drastic differences in these haemocytes from their control counterparts shown in Figures 23 and 24; **a**, fused pair of podocytes; **b**, vermiform cells; **c**, haemocytes agglomerated or clumped to form nodules; some vermiform cells apparently involved; **d**, plasmatocytes with many fine extensions; **e**, apparent cystocyte that is coagulocytelike.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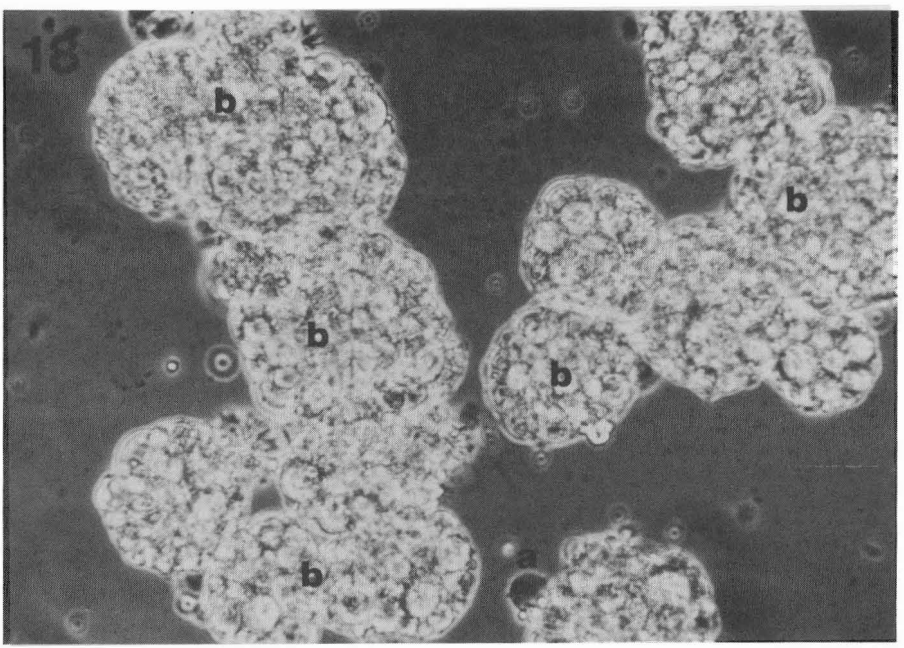
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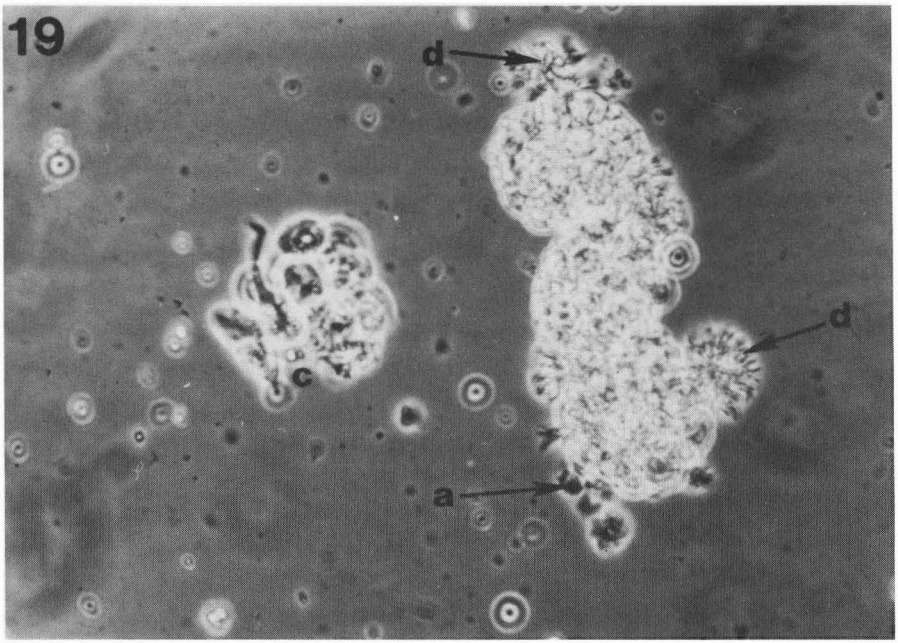
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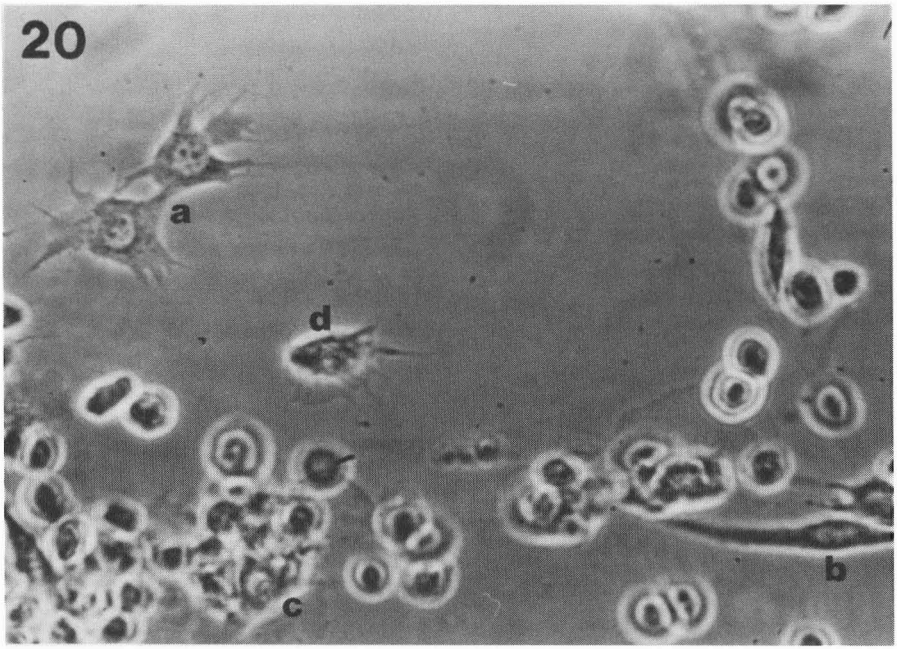
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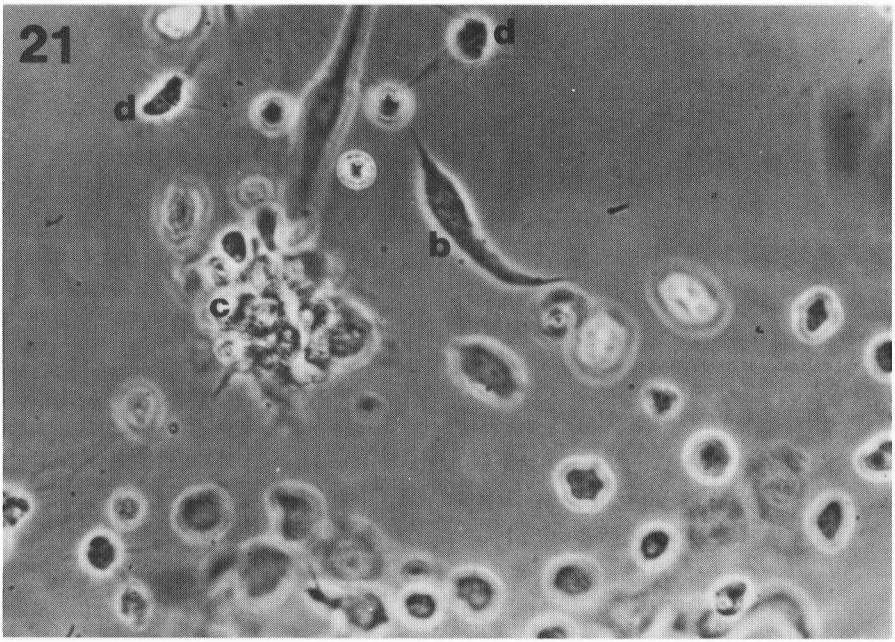
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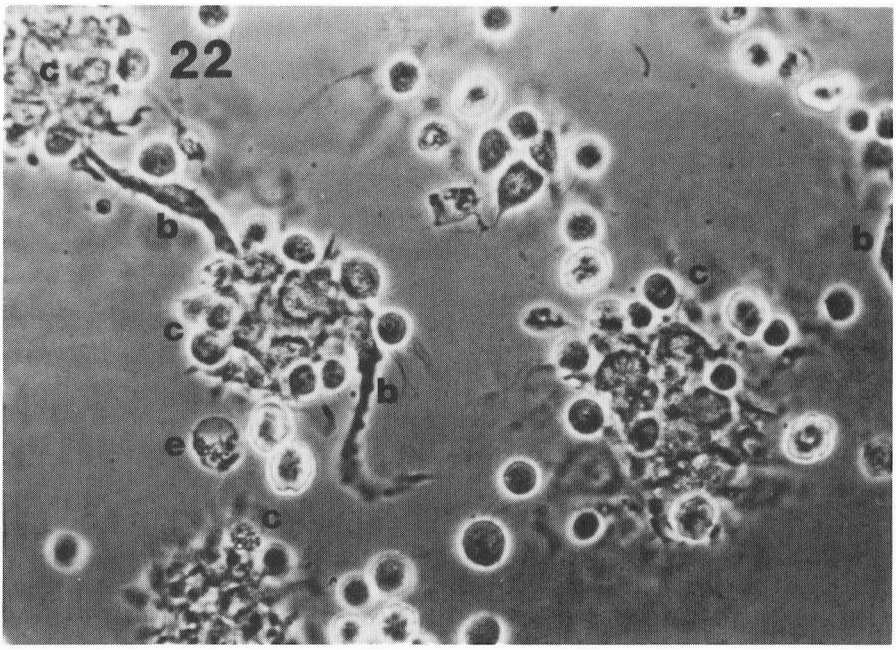
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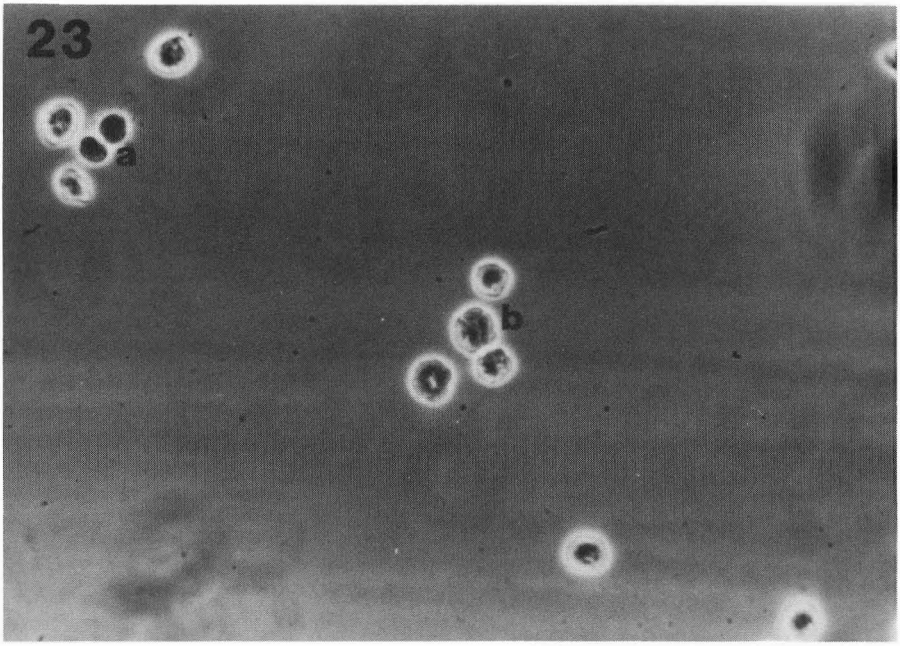
Similar observations also were made with armyworms that were axenically reared on a diet medium without added formaldehyde as an antimicrobial agent. The precocious development of haemocytes in these armyworms may be seen in Figures 25 and 26. The development of haemocytes in these armyworms appears even more advanced than development in the previously described armyworms surviving treatments of pathogens. Compared to the haemocytes in control armyworms of the same age but reared on a diet medium with formaldehyde (Figures 23 and 24), these haemocytes appear in greater numbers and have extensive aggregations and fusions, apparently to form lamellate tissues in some areas. Most of the cells that are fusing are plasmatocytes; other individual cells, such as prohaemocytes, cystocytes, podocytes, and adipohaemocytes may be observed. Individual plasmatocytes with many fine, filamentous extensions also are evident.

The advanced development of haemocytes in quantity, form, and activity also was observed in armyworms infected by exposures to lethal doses of pathogens but physically intact enough to be bled. Figures 27 through 30 are photomicrographs of haemolymph samples showing infected haemocytes from morbid armyworms that were treated with both NPV and *Nosema* in combinations of different formulations. In these haemolymph samples, positive infections by only the virus were observed. Much of the precocity of haemocytes, as already described, is obscured by the virus infection. However, in spite of the abundance of virus inclusion bodies in the samples, the fusions of aggregates of blood cells, the granulations, the vacuolations, and the phagocytoses of larval tissue fragments in individual and complexes of haemocytes may be observed in these photomicrographs. The haemocytes are from larvae that were about 22 days old and are at a much more advanced state of development than would ordinarily be observed

Figures 23, 24. Haemocytes in 19-day-old, control armyworms. Phase photomicrographs, 400x. Note lack of cells fusing and clumping. Cells are low in quantity and appear as individual entities; **a**, prohaemocytes; **b**, adipohaemocytes; **c**, cystocytes.

Figures 25, 26. Haemocytes in 19-day-old armyworms reared on diet medium without formaldehyde. Phase photomicrographs, 400x. Same age as controls shown in Figures 23 and 24; **a**, fusiform plasmatocyte; **b**, extensive fusions of plasmatocytes and podocytes; **c**, plasmatocytes with many fine cytoplasmic, filopodial extensions; **d**, fused aggregation of plasmatocytes to apparently form lamellate tissues.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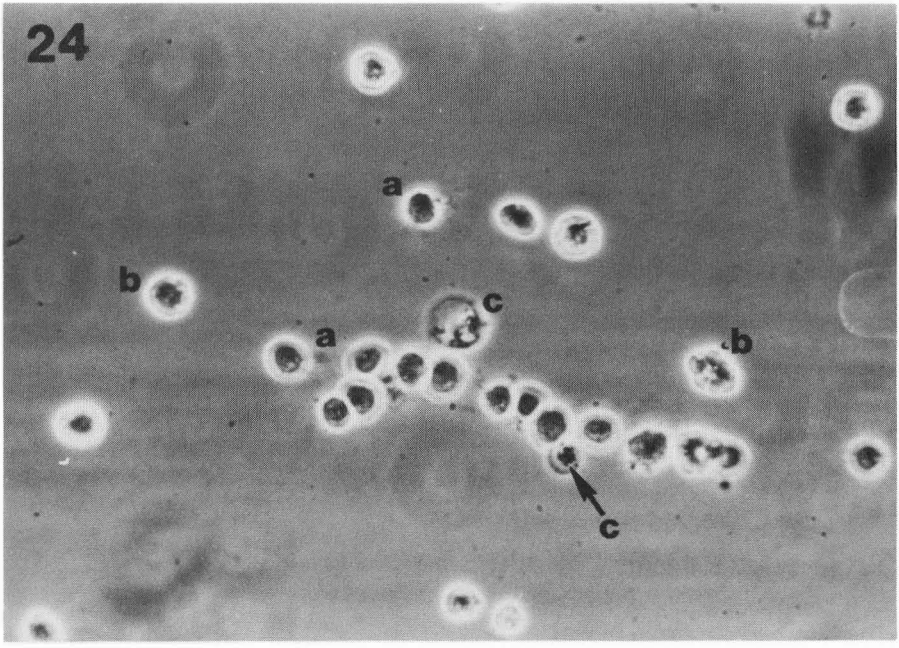
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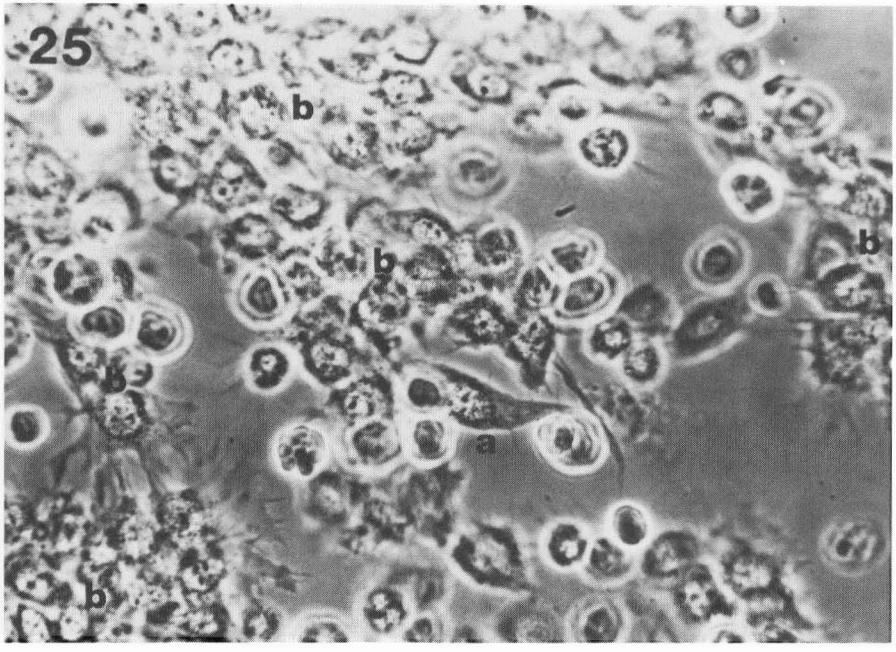
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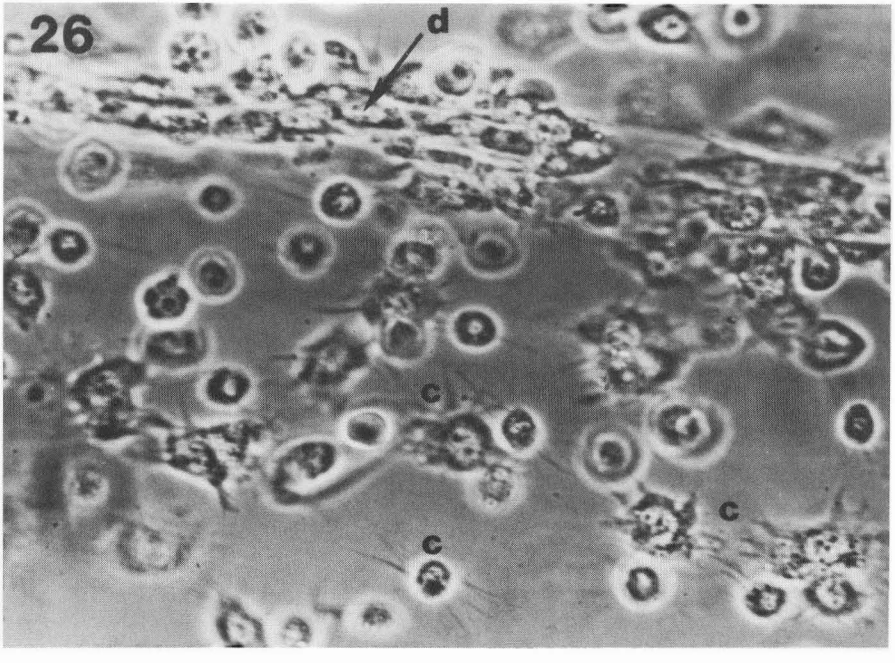
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in control larvae of this age. Extensive degenerative vacuolation and granulation, larval tissue fragmentation due to histolysis, and phagocytosis are processes that all are usually seen in haemocytes during the prepupal (27- to 28-day-old larvae) and early pupal periods for *S. mauritia.* The fragmentation of larval tissues and subsequent phagocytosis of these tissue fragments by haemocytes are rarely observed prior to the prepupal stage. The unusually advanced state of haemocyte development in these haemolymph samples is believed to be due to the combined effects of the two pathogens on the host—NPV as an infectious agent and *Vairimorpha* as a stress producing factor.

Figure 31 is another photomicrograph of an NPV-infected haemolymph sample showing the same kind of advanced development in haemocytes. In this case the infection was due to a low but lethal dose of virus alone. The haemolymph came from morbid, intact, 21-day-old larvae. Again, there is advanced, extensive aggregation of haemocytes forming fused complexes and all the activities associated with degeneration and changes of the prepupal period.

SUMMARY AND DISCUSSION

Examination of haemolymph samples from larvae of different ages and the early pupal stage has revealed that blood cells change in quantity, form, and activity with growth and development of *S. mauritia.* Such changes indicate an apparent metamorphosis of haemocytes in this lepidopterous insect.

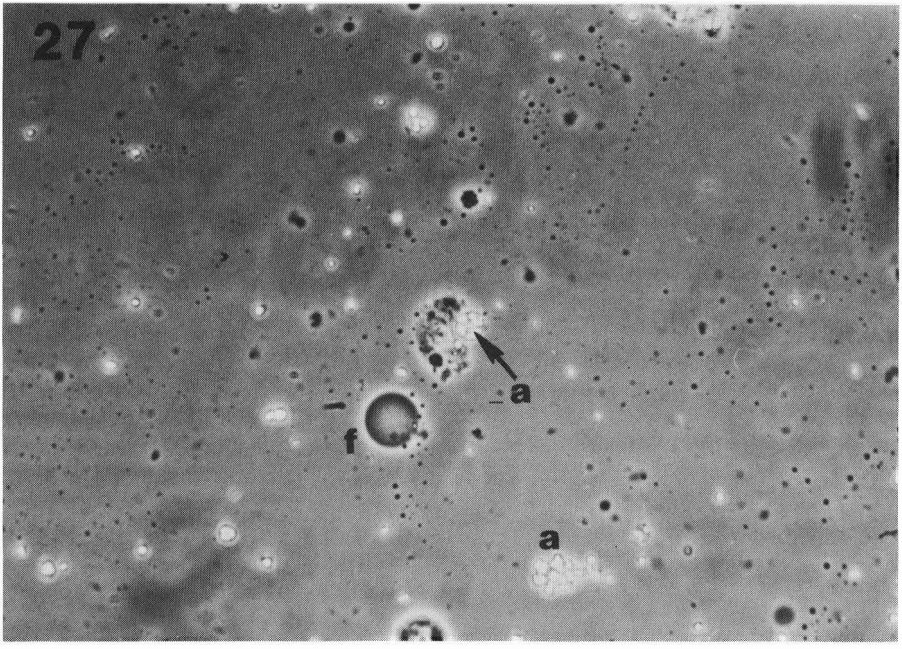
Generally, armyworms that are about 15 days old have individual haemocytes in their blood that are spherical or ovoid and are mainly prohaemocytes, cystocytes, adipohaemocytes, and spherule cells. As growth and development of the armyworm progress, the various types of individual haemocytes increase in numbers, and by the larval age of 20 days, plasmatocytes and oenocytoids also may be differentiated. There have been many studies reporting marked changes in the haemograms, which include THC and DHC, during the development of various insect species. Among these are Yaeger (1945) in *Prodenia eridania* (Cramer), Rizki (1957) in *Drosophila melanogaster* (Meigen), Selman (1962) in *Sialis lutaria* (Linnaeus), Whitten (1964) in *Sarcophaga bullata,* Jones (1967) in *Sarcophaga bullata,* and Bahadur and Pathak (1971) in *Halys dentata.* Usually, haemocyte populations have been

reported to be most abundant during periods of active larval growth and may be observed increasing progressively until just prior to pupation when they reportedly stabilize and then decrease during pupation.

The multiplication and differentiation of haemocytes during the postembryonic development of insects are considered to take place either by (1) mitotic division of existing, circulating cells originating from mesodermal bands in the embryo, or (2) continuous production of cells by haemocytopoietic organs and tissues. Differentiation of haemocytes also may occur by transformations of existing stem cells, such as prohaemocytes. Although the viewpoint with the majority of insects is that multiplication and differentiation of haemocytes are mainly by mitoses of existing cells circulating within hemolymph, in *S. mauritia* these processes are believed to take place by both mitosis and production by haemocytopoietic organs and tissues. During our studies, very few dividing cells were observed compared to the dramatic increases of haemocytes with growth and development, and it was difficult to account for the increases on the basis of mitosis alone. In *S. mauritia,* differentiation also takes place by haemocyte transformations, the occurrences of which were supported by observations of what were believed to be intermediate or transitional forms for these cellular changes. Some of the intermediate forms believed observed were (1) haemocytes during transformations of prohaemocytes to plasmatocytes

Figures 27–30. Haemocytes in morbid armyworms, about 22 days old, that were treated with combinations of NPV and *Nosema;* infection only by virus. Phase photomicrographs, 400x. Highly refractive, small particles free in haemolymph and in haemocytes, virus polyhedra. Vacuolations appear within cell outlines as circles and are not as highly refractive as virus polyhedra. Haemolymph samples show changes in infected haemocytes; **a,** larval tissue fragments; **b,** phagocytozed tissue fragments; **c,** large complex of fused haemocytes manifesting phagocytozed tissue fragments, virus polyhedra and globules of vacuolations; **d,** individual, apparently infected oenocytoid vacuolating; **e,** haemocytes with polyhedra, globules, and tissue fragments; **f,** exudate from degenerating haemocytes; **g,** adipohaemocytes; **h,** substance believed to be a plastid.

Figure 31. Haemocytes in morbid, intact 21-day-old armyworms infected by NPV. Phase photomicrograph, 400x. Note extensive aggregation and fusion of haemocytes; dark granulations more visible in this photomicrograph; **a,** haemocytes within complexes manifesting cell structures with globules and dark granules; **b,** portions of complexes with phagocytozed tissue fragments, virus polyhedra, and degenerative globules.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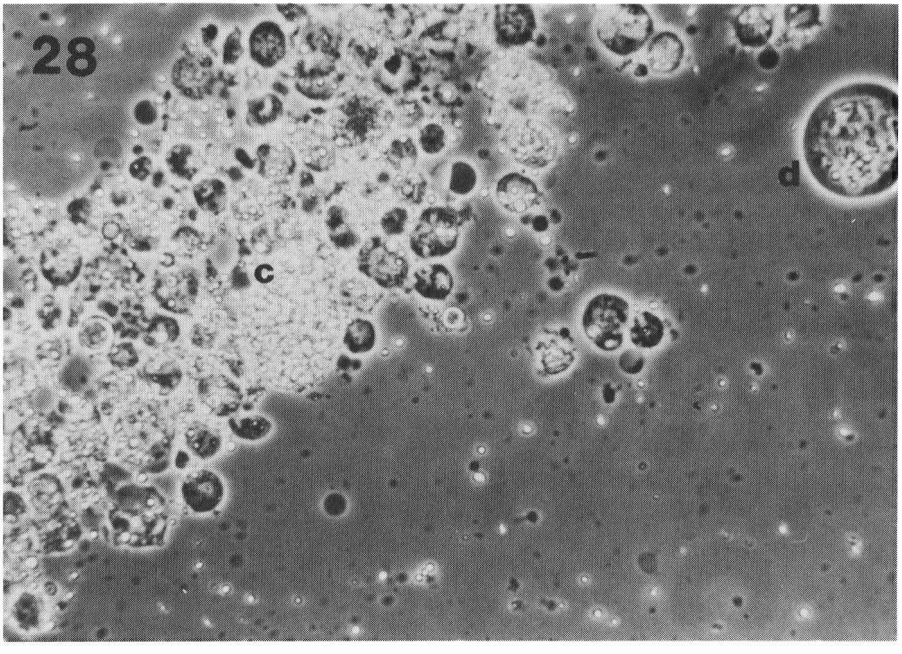
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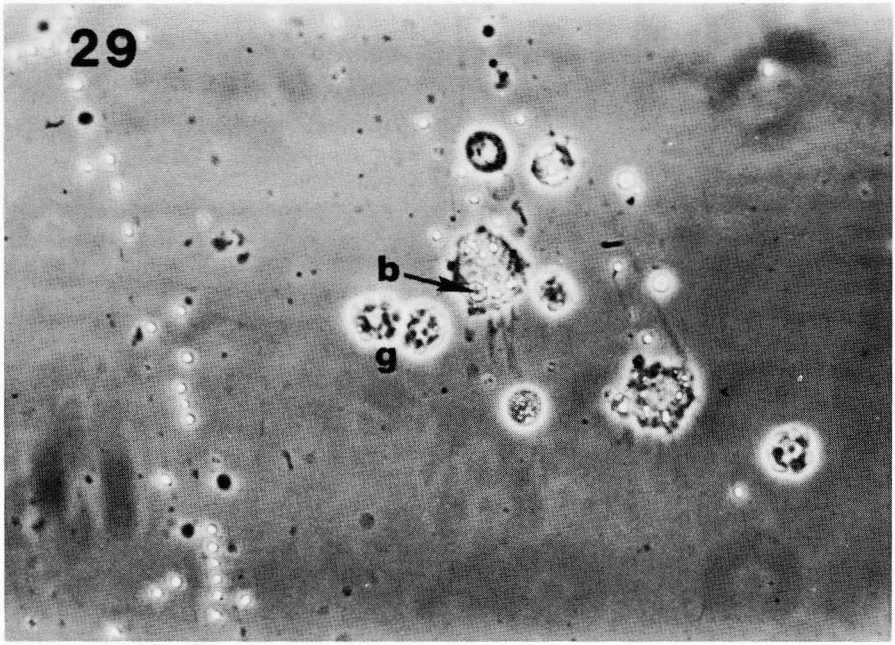
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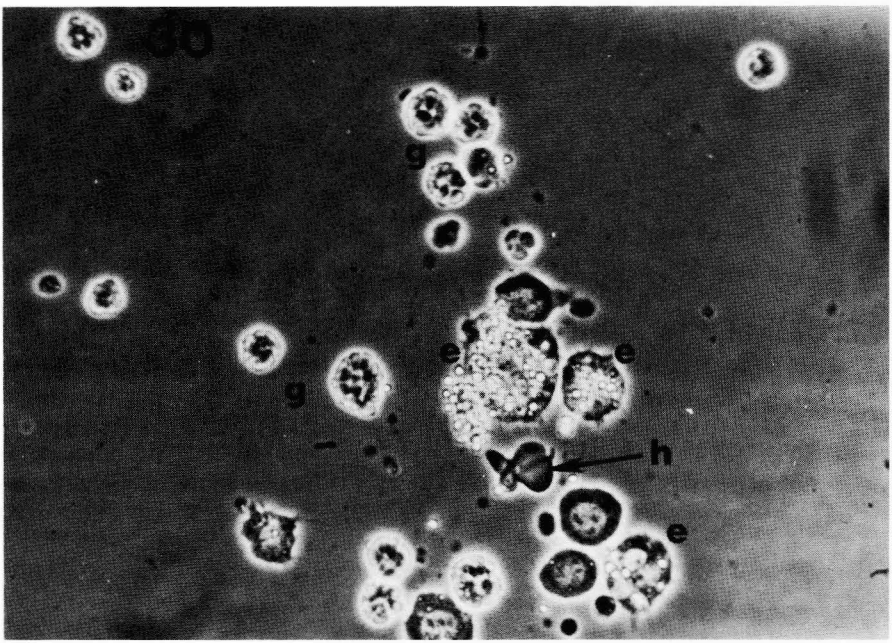
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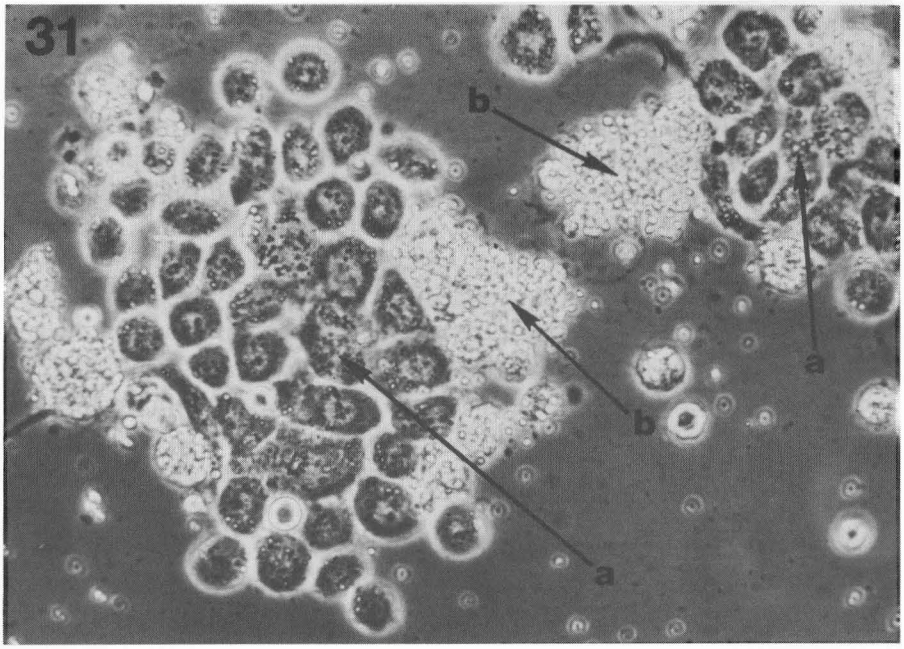

and during plasmatocytes and pleomorphic forms, (2) haemocytes during transformations of prohaemocytes to cystocytes and during cystocytes and hyaline haemocytes, and (3) haemocytes during the transformations of prohaemocytes to spherule cells that seem to occur throughout the developmental period. Generally plasmatocytes accounted for most of the increases in haemocytes during larval growth, with granular haemocytes being predominant just prior to pupation. This same pattern of increases in haemocyte types was observed in *Sarcophaga bullata* by Jones (1967).

The 21- to 26-day-old larval period of *S. mauritia* is characterized by aggregations followed by fusions and agglomerations or clumpings of haemocytes to form complexes and nodules, respectively, composed of many blood cells. Plasmatocytes and granular haemocytes appear to be the types of blood cells mainly involved in the formation of fused aggregates of cells, whereas many different kinds of haemocytes, including plasmatocytes, appear to take part in nodular formations. In addition to the cells observed up to this larval period, vermiform cells, granular haemocytes, podocytes, and hyaline haemocytes begin to appear, and the haemocytes are no longer just floating freely in the

haemolymph. Instead, their behavior and activities (aggregations, fusions, agglomerations) appear to be functional and fit into a scheme of metamorphosis.

The late larval period (26- to 28-day-old larvae) is characterized by processes believed to be the start of degeneration of individual and complexes of haemocytes accompanying metamorphosis. Granulations and vacuolations giving rise to small lipidlike globules and larger spherical blebs may be observed, and the blood cells begin phagocytozing larval tissue fragments resulting from hystolysis. Besides these activities in haemocytes, granular haemocytes become more numerous, individually as well as in complexes, and more cellular transformations occur. Many haemocytes may be seen, particularly plasmatocytes with increased cytoplasmic extensions and pseudopodia, as well as other forms of plasmatocytes and other blood cells transforming to hyaline haemocytes.

The degeneration of cells continues until in 1-day-old pupae the individual and complexes of haemocytes may be seen filled and swollen with globules, granules, and phagocytozed tissue fragments, and in some cases the disruption of cells occurs with extrusion of their inclusions into the plasma. By the time pupae are 3 days old, the activities have progressed to an extent that the phagocytozed tissues completely fill the cells and, along with all the other materials that have accumulated within the cells, transform into highly refractive spheres or balls that dominate the haemolymph. At this point in the pupal stage, only the outlines and some remnants of haemocytes may still be seen, and the haemolymph, by normal vision, has a mushy appearance. Histolysis and phagocytosis are believed to continue within these spheres of haemocytes and tissue fragments to provide some of the metabolic energy and materials necessary for metamorphosis of *S. mauritia,* which includes the formation of new tissues.

The apparent metamorphosis of haemocytes in *S. mauritia* then, involves changes and alterations in activities or processes as well as as in quantity and form. Failure to recognize these changes in haemocytes perhaps has resulted in the diverse and varied information concerning insect blood cells in many reports on haemolymph studies dating back to the late nineteenth century.

Although the metamorphic haemocyte changes may be drastic and great, they still may very easily be overlooked or missed unless well-timed, sequential samplings are planned and studied, because the

changes may be rapid, occurring within 24 hours or less. In this work, the investigations of *S. mauritia* haemolymph under synxenic and varied stress producing conditions enabled observation and recognition of changes in haemocytes ordinarily not easily noticed during different times or ages of the larval and pupal stages. Further haemolymph investigations through the entire pupal and adult stages should prove to be beneficial.

The importance of recognizing the apparent metamorphosis in haemocytes during haemolymph investigations cannot be overemphasized. This recognition and acceptance undoubtedly will result in eliminating some of the variation and diversity in data collected for insect blood cells that has been great enough to cause anomalies from one species to another and within given species.

Of interest among the many studies describing haemocytes are those by Jones (1956, 1962), Rizki (1960, 1962), and Whitten (1964). Their work suggests the possibility of realizing more order and consistency in the data being collected for insect haemolymph. The work by Whitten is particularly detailed, reporting the changes that occur in haemocytes during larval growth, development, and metamorphosis. Her descriptions for haemocyte changes and alterations in *Sarcophaga bullata, Drosophila melanogaster,* and other cyclorrahphous Diptera were similar to those observed in this work for *S. mauritia.* It appears, because of the parallel of the Whitten study and the present work, along with the independent studies by Jones and Rizki, that all insects with similar metamorphoses may possibly have the same scheme of changes in haemocytes during larval growth, development, and metamorphosis. By this suggested correlation, all holometabolous insects or all insects of the same complete metamorphic type should have similar patterns of changes involving identical or closely related blood cell types. In holometabolous insects, such changes in haemocytes should be significant during metamorphosis because the changes and differentiation of tissues from the immature larval stage through the adult stage are significant. On the other hand, with paurometabolous insects or insects of the gradual or simple metamorphic type, the variations and changes in haemocytes accompanying growth and development should not be as great and should be more in quantity, rather than in form and activity, since the morphological changes of these insects from the immature stage through the adult stage are not as dramatic and are essentially only changes in size. Much of the confusion about

haemocytes may be attributed to the variations reported between species and other taxa of insects. However, classifying haemocytes into categories of metamorphic types while conducting investigations should limit many of the apparent variations and consequently the confusion.

Lawn armyworms that have been subjected to treatments of pathogens and other stress-causing influences clearly manifest blood cell changes in their haemolymph. The changes are no different from those observed during usual growth and development, and they are changes in quantity, form, and activity of haemocytes. However, the apparent metamorphosis of haemocytes observed in armyworms subjected to stress treatments occurs much earlier in the insect's life cycle than the metamorphosis of haemocytes usually observed in untreated armyworms. Increased numbers of blood cell types aggregate and agglomerate prematurely to form haemocyte complexes and nodules while haemocytes are vacuolating and granulating. Also, haemocyte forms that would ordinarily be observed in older or more mature larvae may be observed in these treated armyworms. In effect, the host insect apparently reacts to stress-producing factors by advancing the growth and development of its haemocytes to a more mature state, while all other characteristics of the insect appear no different from those of untreated specimens.

Precocious development of blood cells has been reported in tumorous larvae by Rizki (1957*b*). The aggregation and fusion of haemocytes to form syncytia that encapsulate parasites as a defense reaction by parasitized host insects have been reported by Salt (1963) and Nappi and Stoffolani (1971), among other previously cited authors. Salt (1970), furthermore, regards nodule formation as a defense reaction that combines features of both phagocytosis and encapsulation to isolate clumps of bacteria and other foreign particles and that seems to be associated with tumorlike lesions. Such considerations of defense reactions and precocity in haemocytes for insects exposed to microbial pathogens and other stress-producing factors and influences are not known to have been reported yet. However, Tauber (1940) has reported that haemocytes of the roach, *Blatta orientalis* (Linnaeus), become vacuolated, stain more easily and more deeply, and become "ragged" with irregular edges with the progress of an internal bacterial infection. There have been many other reports of phagocytosis as a cellular defense reaction against microbial pathogens. Whitten (1964) feels that phagocytosis plays an important part in removing autolyzed

tissues and cellular debris during growth and metamorphosis.

From the results of studies in this work, the premature aging or advanced development of haemocytes is believed to be part of the defense reactions of *S. mauritia* to disease and stress-producing factors and other influences. Generally, phagocytosis and encapsulation are well established and accepted functions of haemocytes, particularly as defense reactions or as cellular immune responses; they also play a major role in the normal growth and development of the armyworm. They usually take place late in the larval stage, though, and would not be available to function defensively in disease situations during the early larval periods. However, by intrinsically advancing and hastening growth and development in its haemolymph, the armyworm is believed to allow phagocytosis and encapsulation, among other haemocyte activities associated with aging, to function as defense reactions earlier in its larval stage when these processes are needed. This correlation of defense reactions and premature development in haemocytes may most likely be applied to other holometabolous insects.

The precocious development of haemocytes as a defense reaction is supported by the fact that it is manifested by all surviving specimens known to be diseased or under stress, and it occurs without affecting the normal durations of the larval period and the life cycle of the armyworm. Often other precocious developments are observed that are characterized by shortened larval periods and early pupations, but these developments, which also were observed in *S. mauritia,* are not to be confused with the defense reactions of haemocytes. They are most likely insect physiological responses induced by diseased states, and they ordinarily result in small, damaged, or incompletely formed pupae that are terminal. The precocious development in haemocytes as a defense reaction associated with *S. mauritia* gives rise to completely surviving specimens—apparently normal pupae and normal stages of life cycle. From another viewpoint, a relationship between the defense reactions and the apparent metamorphosis of haemocytes is indicated, and perhaps it may be a reasonable explanation for increased resistance to disease with larval growth and aging observed in this insect as well as in others.

The fusion and agglomeration of blood cells, whether observed in the growth and metamorphosis of control armyworms or prematurely in armyworms subjected to stress-producing factors, appear to involve haemocytes that are usually produced during the life cycle of *S.*

mauritia and are not specially produced haemocytes for these activities. The complexes of fused haemocytes are believed to be similar to the multinucleate haemocytes reported for other insects by Jones (1962), Wittig (1962), and Whitten (1964), and also are believed to be similar to the giant blood cells in the review by Wigglesworth (1965). Also, Jones (1965) reports large granulocytophagous cells in *Rhodnius prolixus* that also may be similar to these complexes of fused haemocytes in *S. mauritia.*

Frequently associated with haemocyte fusions and agglomerations, individually or as part of the complexes, are hyaline haemocytes and other lysing haemocytes some of which are believed to be the coagulocytes of Grégoire (1951, 1974). In *S. mauritia,* coagulation in haemolymph is apparently limited to agglutinations of blood cells as manifested in the haemocyte fusions and agglomerations and possibly some of Pattern II coagulation (Grégoire, 1974) involving cytoplasmic extrusion of many threadlike pseudopodial processes by haemocytes. Apart from some streaks in the plasma that seem to be associated with the cytoplasmic extensions, plasma coagulation reactions, such as gelation and precipitation, were not observed in the haemolymph of *S. mauritia.* Interestingly, though, Grégoire (1974) describes plasma coagulation or Pattern I coagulation as being preceded by haemocytes vacuolating, phagocytozing, blebing, and disrupting or disintegrating. The hyaline haemocytes observed in the lawn armyworm are believed to be transformed cystocytes, but some of the lysing and disintegrating haemocytes appeared to be either granular haemocytes or oenocytoids. Cystocytes, granular haemocytes, and oenocytoids, however, are often synonomously described in the literature, and thus it is not unusual for these cell categories to be confused. According to Grégoire's system of classification (1955), cystocytes are derived from the transformations of prohaemocytes (the stem cells), and oenocytoids, and granular haemocytes in turn are derived from transformed cystocytes.

A comparison of the precocious development of haemocytes with the precocious increases of certain haemolymph proteins in *S. mauritia* (Takei and Tamashiro, 1975) has revealed that there is some correlation between these two reactions. However, the precocious development of haemocytes appears to be more sensitive than the increases in haemolymph proteins, occurring, in haemolymph from armyworms subjected to stress-producing factors or other influences, when protein increases do not. However, the possibility still exists that the increases

in haemolymph proteins may be produced by haemocytes at a more advanced period of aging, most likely to a degree where cell degeneration is more pronounced. Perhaps the recognition of precocious development in haemocytes may be the most sensitive means of determining early or latent infections and other disease situations in apparently normal armyworms.

While the direct correlation between premature increases in haemolymph proteins and the premature developments in haemocytes remains unsure, the two defense reactions were always elicited. This happened with differing sensitivities, no matter what the stress-producing factors and influences were, whether a biologically infectious agent or whether a purely physical factor. In the haemolymph studies involving lawn armyworms reared on a diet not containing formaldehyde, the advanced developments in haemocytes give further support to the hypothesis by Takei and Tamashiro (1975) that the host insect in Hawaii is harboring a slow-acting disease that may be inhibited by formaldehyde. The disease, believed to be a chronic viruslike infection that is endogenous to the host, also elicits the premature increases in certain haemolymph proteins. Thus, both defense reactions by the host are elicited by this apparent infection just as they are in specimens subjected to stress-producing conditions.

Finally, it must not be overlooked that these studies of haemolymph in *S. mauritia* suggest a relationship between the defense reactions of this insect to stress and its endocrine system. Stress appears to affect the endocrine system of the larva in such a way that out of all the many possible, intricate physiological processes related to the endocrine system, only those controlling the development in haemolymph appear to be involved. The concept of stress affecting the endocrine system of animals is not new, and Selye (1950, 1952, 1955) has recognized the role of stress in the diseases of man. According to Steinhaus (1958), the central core of Selye's concepts deals with the endocrine system and its response to various "stressors" (stress-producing factors and influences). Selye (1973) continues to report on the concepts surrounding stress, disease, and adaptation. In insects, Ben-Shaked and Harpaz (1966), have reported the close association of latent virus infections in *Prodenia litura* (Fabricius) with an endocrine mechanism regulating growth and development.

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